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Cloning and characterization of a novel endothelin receptor subtype in the avian class

(neural crest/development/melanocytes)

LAURE LECOIN*, TAKESHI SAKURAI†, MINH-TRIET NGO*, YOICHIRO ABE‡, MASASHI YANAGISAWA†, AND NICOLE M. LE DOUARIN*§

*Institut d'Embryologie Cellulaire et Moléculaire du Centre National de la Recherche Scientifique et du Collège de France, Nogent sur Marne, France; †Howard Hughes Medical Institute and Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX 75235-9050; and ‡Institute of Basic Medical Sciences, University of Tsukuba, Ibaraki 305, Japan

Contributed by Nicole M. Le Douarin, January 5, 1998

ABSTRACT **Endothelin 3 (EDN 3) and the endothelin receptor B (EDNRB) are involved in the development of neural crest and particularly of the melanocytes and the enteric nervous system. We reported previously that the avian *EDNRB* gene is expressed in the neural fold before crest cell migration and later on in all the neural crest derivatives except, at any developmental stage, in the melanocytic lineage. However, quail melanoblasts proliferate in response to EDN 3 stimulation *in vitro*. These observations prompted us to search for another type of endothelin receptor (EDNR). We report here the cloning by reverse transcriptase–PCR of an avian cDNA encoding a subtype of EDNR, which we have called EDNRB2, because its deduced amino acid sequence is more closely related to that of EDNRB than to either the mammalian EDNRA or to the *Xenopus* EDNRC. Its expression pattern differs from that of the "classical" avian *EDNRB* because it is strongly expressed in melanoblasts and melanocytes. *EDNRB2* transcripts are also abundant in the liver and kidney. Our pharmacological studies showed that EDNRB2 binds with similar affinity to EDN 1, EDN 2, and EDN 3, further confirming that this receptor belongs to the B type, although it displays a low affinity for sarafotoxin-c, a known EDNRB-selective agonist.**

When neural crest (NC) cells leave the neural primordium and begin to migrate, most of them are pluripotent. Their developmental potentialities become progressively restricted as migration proceeds and as they home to the target tissues where they reach their fully differentiated state (1–7).

A large body of evidence has demonstrated that the commitment of NC cells to definite developmental fates greatly depends on the environmental cues they encounter during and at the end of their journey (8, 9). Identification of the environmental factors influencing NC cell development has progressed considerably in recent years. Clues in the search of these factors have been provided by the cloning of genes responsible for mutations that affect NC derivatives in the mouse. Such was the case for two distinct mutants exhibiting similar phenotypes, Piebald lethal (s^l) and lethal spotting *(ls)*, both of which are characterized by alterations in pigment cell and enteric nervous system development. The identification of the s^l and *ls* gene products revealed that these genes encode, respectively, a G-coupled-heptahelical transmembrane receptor and its ligand (10, 11). The ligand belongs to a family of highly conserved 22-aa peptides that includes three members: endothelin 1, 2, and 3 (EDN 1, -2, and -3). These peptides result from a two-step proteolytic processing of larger precursors called preproendothelins 1, 2, and 3 encoded by three different genes (12, 13). To date, two types of endothelin receptors (EDNR) have been identified in higher vertebrates: EDNRA, which binds EDN 1 with high affinity, and EDNRB, which displays similar affinities for the three types of endothelins. In *Xenopus*, a third type of EDNR, referred to as EDNRC, has been cloned from dermal melanophores (14). No *EDNRC* homolog has been identified in Amniotes.

The mode of action of EDN 3 on NC derivatives has been investigated on cultured quail NC cells by Lahav *et al.* (15), who showed that EDN 3 exerts a potent mitogenic effect on these cells. Moreover, *in vivo*, NC cells express *EDNRB* from the premigratory stage onward in all crest derivatives except for cells of the mesectodermal and the melanocytic lineages (16). In addition to its dramatic effect on NC cell proliferation, EDN 3 was found to increase considerably the proportion of crest cells differentiating into melanocytes in culture. The fact that neither melanoblasts nor melanocytes expressed EDNRB *in vivo* or *in vitro* seemed paradoxical. We therefore decided to search for a third type of receptor that binds EDN 3 and is expressed in the cells of the melanocytic lineage. We report here the cloning, expression pattern, and pharmacological properties of such a receptor in quail and chicken species. This receptor, which is closely related to EDNRB structurally and pharmacologically, is designated as EDNRB2.

MATERIALS AND METHODS

Reverse Transcriptase–PCR (RT-PCR) and Cloning. Skin from the neck and back region of embryonic day 6 (E6) quail embryos was peeled off, and tissues were dissociated with pancreatin (GIBCO). The epidermis was removed and subjected to a trypsin/EDTA solution, and 2×10^6 cells were cultured for 1 week in DMEM + 10% FCS + 2% chicken embryo extract and 100 nM EDN 3, a culture medium that favors melanogenesis (15). At the end of the culture period, most cells were lightly pigmented, indicating their engagement in the melanogenic differentiation pathway. Total cellular RNAs were isolated according to the method of Chomczynski and Sacchi (17), which was modified slightly: cells were lysed directly by guanidium thiocyanate solution. Ten micrograms of total cellular RNA was reverse transcribed with 200 units of Superscript II reverse transcriptase (GIBCO). The single-strand cDNA synthesis was primed with 50 pmol of the 3′ oligonucleotide used in the PCR experiment. The primers used for PCR were two degenerate oligonucleotides designed on the

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Abbreviations: NC, neural crest; EDN, endothelin; EDNR, endothelin receptor; RT-PCR, reverse transcriptase–PCR; E, embryonic day; TM, transmembrane domain; Z, stage of Zacchei (ref. 21).

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. Y16089).

§To whom reprint requests should be addressed. e-mail: ledouari@infobiogen.fr.

basis of the sequence of transmembrane domains V–VII of rat (18), bovine (19), and quail *EDNRB* (16) and of *Xenopus EDNRC* (14) receptors. The sequences of the 5′ and 3′ oligonucleotides were respectively 5′-GGTGGCTST-TYRGNTTYTATTTC-3′ and 5′-TGCTSACMAAR-TASAGWGC-3′. The PCR was performed in a total volume of 100 μl comprising half of the RT reaction mixture (20 μl), 1.3 mM $MgCl_2$, 0.5 mM dNTP, 50 pmol, and 100 pmol of the 3′ and 5′ primers, respectively, 5 units of Ampli *Taq* polymerase (Perkin–Elmer) and the buffer according to the manufacturer's instructions. Thirty cycles of PCR amplification were performed in a Perkin–Elmer Cetus DNA thermal cycler with the following program: 94°C for 1 min, 45°C for 30 sec, 72°C for 1 min, and a last cycle in which the elongation phase at 72°C lasted 7 min before holding at 4°C. A 260-bp PCR product was purified on a 2% NuSieve low-melting agarose, subcloned into a pCRII vector with a TA cloning kit (Invitrogen), and sequenced.

Approximately 10^6 recombinants of an E4 quail cDNA library were screened with this PCR-generated fragment that was ^{32}P-labeled with a random labeling kit (Promega), as previously described (20). The filters were hybridized at high stringency (42°C, 50% formamide) and washed at 65°C in 0.1× SSC, 0.1% SDS. One clone of 3.5 kb was isolated. A 1.7-kb *Sac*I fragment that hybridized with the PCR insert was subcloned into PGEM vector and sequenced by Genome Express. The remaining 5′ fragment of the original clone was then sequenced with a Sequenase version 2.0 kit (United States Biochemical). The amino acid sequence, hydropathy analysis, and sequence alignments were performed with the GENEWORKS program (IntelliGenetics).

***In Situ* Hybridization.** Quail eggs obtained commercially were incubated at 38°C and staged according either to the number of days in incubation (E*n*) or, more precisely, according to Zacchei (Z) (21). Whole quail embryos were fixed at every stage ranging from E2 (24-somite stage) to E9 in Carnoy's fluid. At E10 to E14, only the dorsal skin was fixed. Paraffin-embedded tissues were serially sectioned at 7.5 μm. The slides were treated as previously described (22).

To generate antisense and sense probes, the full 3.5-kb clone was linearized with *Hin*dIII and *Eco*RV, respectively, and transcribed with sp6 and T7 RNA polymerase with the Promega Riboprobe kit. Hybridization at high stringency (52°C, 50% formamide) and washings (65°C) were performed according to Wakamatsu and Kondoh's method (23) modified as described in ref. 22. In these conditions there is no cross-hybridization of *EDNRB2* with *EDNRB* mRNA-containing cells.

Pharmacological Characterization of Quail EDNRB2. A full-length *Xho*I-*Not*I insert of the quail *EDNRB2* cDNA was directionally subcloned in the pME18Sf-expression vector (24). For radioligand-binding assay, Ltk$^-$ cell monolayers in 10-cm dishes (2×10^6 cells per dish) were transfected with the quail *EDNRB2* or human *EDNRB* (24) expression plasmid by the DEAE–dextran method. One day after transfection, cells were seeded in 24-well microtiter plates at 10^5 cells per well. After an overnight culture, monolayers of cells were washed twice and incubated with 20 pM [^{125}I-Tyr^{13}]EDN 1 in the presence of various concentrations of competitors or 1 h at 22°C as previously described (25). After extensive washing, cells were lysed in 0.1 M NaOH and cell-bound radioactivity was determined by a gamma counter. For intracellular Ca^{2+} transient analysis, Ltk$^-$ cells in 10-cm dishes (2×10^6 cells per dish) were similarly cotransfected with an α_{1B} adrenergic receptor cDNA [pcDV1Ra1B (26)] and either the quail *EDNRB2* or human *EDNRB* cDNA. Two days after transfection, cells were dispersed by trypsinization and loaded with Fura 2-AM in solution A (140 mM NaCl/4 mM KCl/1 mM Na_2HPO_4/1 mM $MgCl_2$/1.25 mM $CaCl_2$/11 mM glucose/5 mM Hepes, pH 7.4/0.2% BSA) for 30 min at 37°C as previously described (24). Fura 2-loaded cells were washed and resuspended in solution A and stimulated with designated concentrations of endothelin agonists in a 500-μl glass cuvette. Cellular fluorescence was measured with excitation at 340 nm and 380 nm and emission at 500 nm with a CAF-110 Intracellular Ion Analyzer (Jasco, Easton, MD). Peak intracellular Ca^{2+} concentrations during Ca^{2+} transients evoked by endothelin agonists were normalized by peak Ca^{2+} concentrations evoked in the same cell suspension by 10^{-5} M noradrenaline. Further analyses using various endothelin receptor antagonists will be required for a complete pharmacological characterization of the receptor.

RESULTS

Molecular Cloning of the Endothelin Receptor Expressed in Quail Melanocytes. To identify the EDNR expressed in the melanocytic lineage, RNAs from E6 quail skin cells, cultivated in the presence of EDN 3 for 7 days (15), were subjected to RT-PCR. Sequence comparison of bovine, human, rat, and quail *EDNRB* and of *Xenopus EDNRC* led us to design degenerated oligonucleotides to amplify a fragment situated in transmembrane domains (TM) V–VII (represented between the two arrows in Fig. 1). In this region, the cloned *Xenopus EDNRC* gene displays an additional region of 40 nt, which cannot be aligned with the other cloned *EDNR*s. The expected size of the PCR fragments therefore would be 300 bp if they corresponded to a "C" type and 260 bp if they corresponded to an "A" or a "B" type *EDNR*. A 257-bp fragment was amplified that shared 70% and 83% identity with the quail EDNRB gene at the nucleic acid and deduced amino acid levels, respectively. Preliminary *in situ* hybridization experiments with this PCR product revealed hybridizing mRNA in melanoblasts dispersed in the skin. We then undertook the screening of an E4 quail cDNA library. A single 3.5-kb cDNA clone encompassing the whole ORF was isolated. The deduced amino acid sequence alignment of this receptor with other EDNRs shows that it differs significantly from the previously cloned EDNRB in every domain of the molecule (Fig. 1). Between TM I and the carboxyl terminus, the EDNRB2 amino acid sequence shares an identity of 74% with that of quail EDNRB, whereas quail and mammalian EDNRB share an identity of 90%. EDNRB2 thus does not represent a splice variant of EDNRB but constitutes an endothelin receptor encoded by a different gene. Moreover, EDNRB2 is slightly more closely related to EDNRB than EDNRA (59 and 55% identity to rat EDNRB and EDNRA, respectively). Sequence similarities vary according to the region of the molecule considered: the EDNRB2 extracellular domain differs significantly from that of the other known EDNR. In the region between TM I–IV, EDNRB2 is closer to rat EDNRB (83% identity) than rat EDNRA (71%) or *Xenopus* EDNRC (66%). In TM V and VI, EDNRB2 displays more identities with EDNRA (91 and 95%, respectively) than with EDNRB (82 and 83%), and, very strikingly, in TM VII to the carboxyl terminus, EDNRB2 has a high degree of homology with *Xenopus* EDNRC (83% identity with EDNRC in the last intracellular domain, compared with 40–53% identity with the same domain of EDNRA and EDNRB, respectively).

Pharmacological Properties of the Quail EDNRB2. The predicted sequence of the quail EDNRB2 was only marginally more similar to mammalian EDNRB than to EDNRA, raising the possibility that the pharmacology of this receptor could be significantly different from either of the mammalian receptors. We examined the selectivity of the EDNRB2 against a panel of natural endothelin agonists by using competitive radioligand-binding assays as well as intracellular calcium transient assays. An expression vector encoding the full-length *EDNRB2* was transiently transfected into Ltk$^-$ cells. Human *EDNRB* cDNA was similarly expressed in Ltk$^-$ cells and used as a

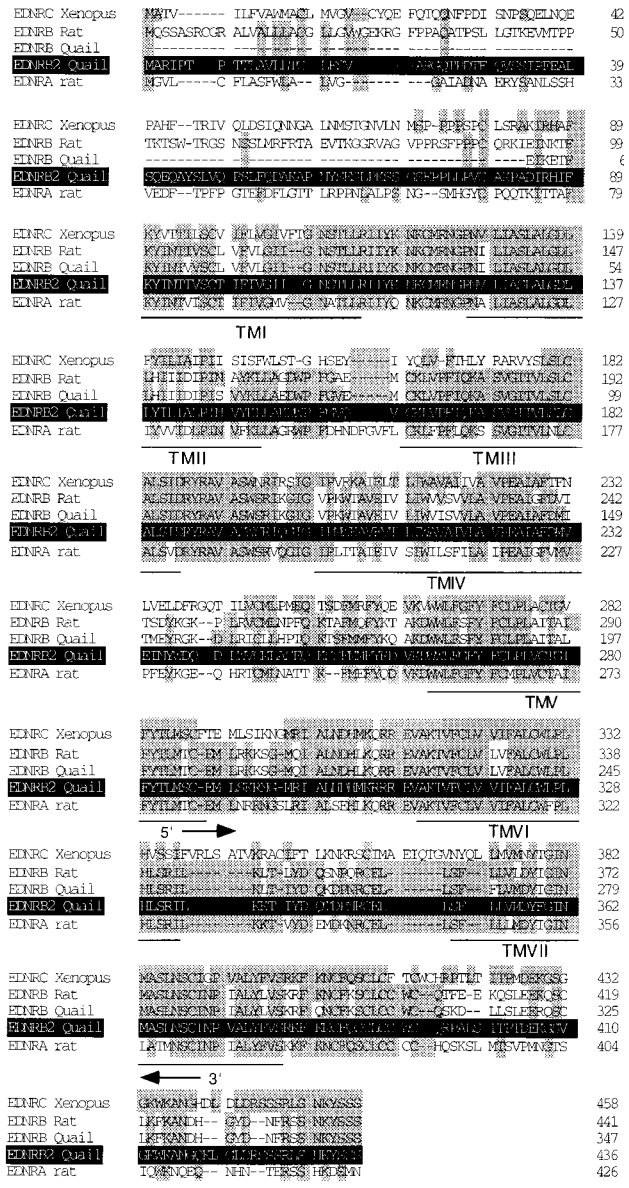

FIG. 1. Amino acid alignment of the quail EDNRB2 (in white on a black background), quail EDNRB (16), rat EDNRB (18), *Xenopus* EDNRC (14), and rat EDNRA (43). The residues in common between EDNRB2 and other endothelin receptor are presented on a gray background. Transmembrane domains are underlined. The region of the molecule whose sequence has been amplified by RT-PCR is located between the two arrows (representing the oligonucleotides).

reference. Ltk$^-$ cells transfected with the quail EDNRB2 cDNA showed significant levels of specific [^{125}I]EDN 1 binding, whereas cells transfected with the vector alone exhibited no detectable level of specific [^{125}I]EDN 1 binding. The level of specific [^{125}I]EDN 1 binding without competitors on cells expressing the quail EDNRB2 was similar to that on cells expressing the human EDNRB, indicating that similar densities of the receptors were expressed on the cell surface. Binding assays with nonradioactive EDN 1, -2, and -3 as competitors showed that these ligands had similar apparent affinities against the EDNRB2 at low nanomolar ranges, as they did against the human EDNRB (Fig. 2 *A* and *B*). However, in striking contrast to the human EDNRB, which exhibited almost equally high affinities for sarafotoxins-b and -c as reported previously (27), the quail EDNRB2 showed affinities for sarafotoxins-b and -c that were nearly two and three orders of magnitudes lower, respectively. Calcium transient assays

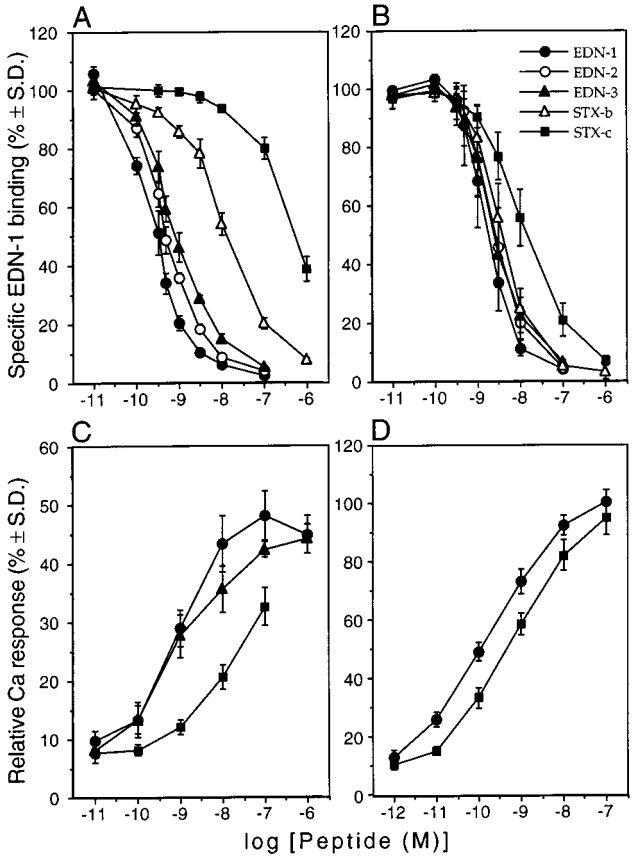

FIG. 2. Pharmacological characterization of quail EDNRB2 receptor expressed in transfected Ltk$^-$ cells. (*A* and *B*) Competitive radioligand-binding assay. Displacement of specific [^{125}I]EDN 1 binding to cells expressing quail EDNRB2 (*A*) and human EDNRB (*B*) by increasing concentrations of unlabeled endothelin-related peptides are determined in quadruplicates. Values are plotted as percentage of the levels of binding without competitors. (*C* and *D*) Dose–response relationships of intracellular Ca^{2+} transients evoked by endothelin-related peptides in cells expressing quail EDNRB2 (*C*) and human EDNRB (*D*). Peak cytoplasmic Ca^{2+} concentrations are determined in quadruplicates and plotted as percentage of responses evoked by 10^{-5} M noradrenaline.

confirmed that EDN 1, -2, and -3 acted as agonists on both receptors and had similar nanomolar EC_{50} values for both receptors (Fig. 2 *C* and *D*). On the human EDNRB, sarafotoxin-c was approximately equipotent with EDN 1 in this assay as reported previously (27). Nevertheless, although sarafotoxin-c did work as an agonist for the quail EDNRB2, its apparent potency was nearly two orders of magnitude weaker than EDN 1 on this receptor.

Expression Pattern of *EDNRB2*. The expression pattern of *EDNRB2* was studied during quail development. Preliminary experiments showed that this *EDNRB2* probe also hybridizes in the chicken embryo (data not shown). The first *EDNRB2* transcripts were found at stage 15Z, in scattered cells of the cephalic mesenchyme occupying the position of migrating melanoblasts (22) and, at the trunk level, in a few NC cells located between the neural tube, dorsal ectoderm, and somite, in the region where melanoblasts were reported to stay before entering the dorsolateral pathway of migration (28, 29), as well as in cells lining the blood sinusoids of the liver. From stage 18Z (E4) onward (Fig. 3 *A* and *B*), numerous labeled cells were observed along the dorsolateral migration pathway of NC cells, underneath the ectoderm, whereas *EDNRB2* expression could not be detected in any other NC derivative along the dorsoventral pathway of migration. Some of the positive cells had

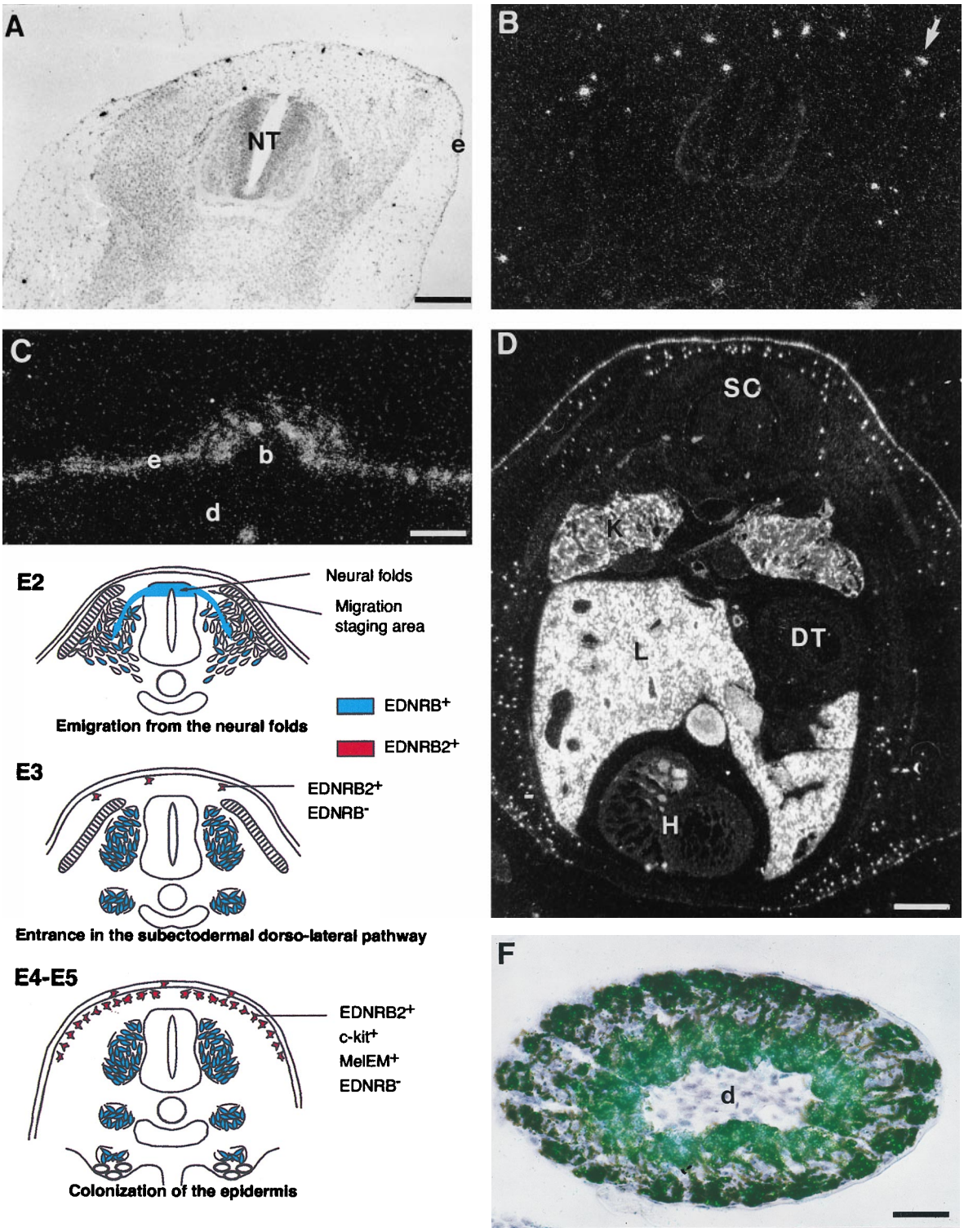

FIG. 3. *EDNRB2* expression pattern in the quail embryo. (*A* and *B*) *In situ* hybridization with *EDNRB2* probe on a transverse section of E4 quail embryos (stage 18Z) showing labeled cells, presumably melanoblasts, migrating under the ectoderm (e). Some of them have already entered the ectoderm (arrow). NT, neural tube. (*C*) Detail of a E6 quail feather bud. Individual cells in the epidermis (e) and in the deep dermis (d) express strongly *EDNRB2*. These cells are especially numerous in the dorsal feather buds (b). (*D*) *In situ* hybridization with *EDNRB2* probe on transverse section of E6 quail embryos (stage 23Z). General view showing a strong hybridization signal in the liver (L), kidney (K), and skin. The white patches in the heart are an artifact due to the blood, visualized in dark field at low magnification. SC, spinal cord; DT, digestive tract; H, heart. (*E*) Schematic representation of the respective expression pattern of the two *EDNRB* genes during avian NC cell development. During emigration from the neural tube, the NC cells express EDNRB, whereas as soon as they become engaged in the dorsolateral pathway, they start expressing *EDNRB2* and stop expressing *EDNRB*. (*F*) *EDNRB2* expression in pigmented melanocytes, as shown by *in situ* hybridization with *EDNRB2* probe on transverse section of E14 quail feather filament. The labeling is concentrated at the basis of the barb ridges near the dermal pulp of the feather filament (d) where the cell bodies of the melanocytes are located. The melanocytes extend processes toward the external keratinocytes and transfer their melanosomes, which form clumps of pigment at the periphery of the feather filament. Bright field (*A*); dark field (*B–D*); and epipolarized illumination (*F*). [Bars = 122 μm (*A* and *B*), 61 μm (*C*), 183 μm (*D*), and 64 μm (*F*).]

already entered the epidermis. The hybridization signal in the liver remained strong over the whole period examined (up to E9). Faint and patchy *EDNRB2* expression became detectable in the kidney at that stage. At stage 23Z (E6) (Fig. 3*C*) dispersed cells in the skin were strongly *EDNRB2* positive, especially in the developing feather buds and in the deep dermis. Moreover, an increasing number of cells were expressing the gene in the kidney. In addition, a very faint hybridization signal could be detected in dorsal root ganglia and Schwann cells lining the nerves (not shown). In contrast to *EDNRB*, expression of *EDNRB2* was never observed in the gut (Fig. 3*D*). At E9 and later, mature, strongly pigmented melanocytes in skin and feathers remained *EDNRB2* positive (as shown in transverse section of an E14 feather filament; Fig. 3*F*).

DISCUSSION AND CONCLUSIONS

In this study, we demonstrate the existence of an endothelin receptor in birds. The *EDNR* type B has been partially cloned previously in the quail using a similar RT-PCR strategy (16). The amino acid sequence of this EDNR significantly differs from quail EDNRB in every region of the molecule and therefore does not represent an alternatively spliced form of the same gene transcript. Furthermore, a cDNA encoding the chicken EDNRA has been recently identified (A. Amemiya and M.Y., unpublished results), the amino acid sequence of which is clearly different from that of EDNRB2. Three types of EDNRs thus have now been identified in birds.

Quail EDNRB2 Exhibits Atypical EDNRB-Type Pharmacology. Our radioligand-binding experiments together with intracellular calcium transient assays on transfected cells confirmed that the quail *EDNRB2* cDNA indeed encodes a functional endothelin receptor polypeptide. The three mammalian endothelin isopeptides, EDN 1, -2, and -3, exhibited approximately equal high (nanomolar) affinities and efficacies on this receptor, qualifying the molecule as a "type B" endothelin receptor (30). This also indicates that quail homolog(s) of any of the three mammalian endothelins can be the physiologically relevant endogenous ligand for this receptor in the melanocytic lineage. However, further pharmacological characterization using endothelin agonists from snake venom, sarafotoxins, revealed that the quail EDNRB2 has low affinities to sarafotoxins, especially sarafotoxin-c. Sarafotoxin-c is well established in the field as a highly selective EDNRB agonist; mammalian EDNRB exhibits high affinity for sarafotoxin-c, whereas the EDNRA shows low (> mM) affinity to this ligand. The quail EDNRB2 behaved more like mammalian EDNRA against sarafotoxin-c. In this regard, EDNRB2 has a pharmacological property that is highly atypical for a type B receptor.

The pharmacological behavior of EDNRB2 may be compared with the receptors characterized pharmacologically in *Xenopus* liver (31) and in the dog (32). Pharmacological properties of this EDNRB2 are in agreement with its deduced amino acid sequence, because transmembrane domains I–III, which are crucial for EDN 1 binding (24, 33), are more similar to EDNRB than EDNRA, whereas the transmembrane domains V and VI, which determine binding to EDN 3 agonists such as IRL1620 (24), are more homologous to EDNRA than EDNRB.

Quail *EDNRB* and *EDNRB2* Display Different Expression Patterns. *EDNRB2* is strongly expressed in the melanocytic lineage from the time NC cells begin to migrate in the skin pathway throughout embryonic development and in fully mature melanocytes as well. *EDNRB* expression is excluded from this cell lineage. *EDNRB2* is not expressed in the other NC derivatives at any developmental stage except at a low level in dorsal root ganglia and Schwann cells. The NC precursors strongly express *EDNRB* in the neural folds and continue to do so as they undergo the epithelio-mesenchymal transition and yield the migratory NC. Thus, all the NC derivatives (peripheral ganglia, Schwann cells, adrenomedullary cells) express *EDNRB* throughout quail and chicken development with the exception of the melanocytes and mesectoderm (16). We show here that, in birds, the former express a unique type of EDNR, which is also expressed mainly in cells lining the blood sinusoids of the liver and in a so far undetermined cell type(s) in the developing kidney. Development of the mesenchymal derivatives of the NC is also dependent on endothelins because the targeted mutation of EDN 1, EDNRA, and endothelin-converting enzyme 1 (34–36) in the mouse strongly alters the development of mesectodermal derivatives. The ontogeny of all NC derivatives, therefore, depends on endothelin signaling with different receptors. The potent mitogenic effect of EDN 3 was reported previously (15). When added to cultures of quail NC cells, EDN 3 first stimulates cell proliferation while inhibiting cell differentiation. After day 5 of culture, however, differentiation of melanocytes is strongly stimulated. NC cells express high levels of *EDNRB* at the onset of the culture but cease to do so when they enter the melanogenic differentiation pathway. Similarly, *in vivo*, all the crest cells express *EDNRB* except those migrating to the skin. On the basis of previous studies by our group, one can establish the appearance of melanocytic molecular markers according to the sequence indicated in Fig. 3*E*: *EDNRB2* is expressed in quail melanoblasts at E3, just before the MelEM antigen that appears in early melanoblasts (37) and the c-*kit* receptor (22) that can be detected in the skin pathway at E3.5–E4 only. We thus propose that EDN 3 first exerts a strong proliferative activity on early NC precursors (15) that are mostly pluripotent (refs. 1 and 38; R. Lahav, personal communication). This mitogenic effect of EDN 3 is mediated by EDNRB. When the cells become engaged in the melanocytic pathway, they switch to EDNRB2 (Fig. 3*E*). *In vitro* experiments are in progress to determine whether such a switch can be induced in culture.

These observations raise the question as to whether *EDNRB2* has a homolog in mammals. Interestingly, in contrast to birds, *EDNRB* has been shown to be expressed in mouse melanoblasts (M.Y., unpublished results). However, several pharmacological studies suggest the existence of additional EDNR of either the A or B subtype. Atypical PD142893-insensitive EDNRB has been found in the rabbit pulmonary artery (39), and a PD145065-insensitive EDNRB in the guinea pig ileum (40). EDNRB-like receptors insensitive to sarafotoxin-c have been reported in rat (41) and *Xenopus* liver membranes (31), and both RES 701–1-sensitive and -insensitive EDNRB have been described in rat kidney (42). Similarly, an IRL 1620-insensitive EDNRB has been reported in dog (32). No molecular data are available so far concerning these receptors.

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